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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Method for high throughput cell-based assays using versatile living microarrays

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METHOD FOR HIGH THROUGHPUT CELL-BASED ASSAYS USING VERSATILE LIVING MICROARRAYS

Field of the Invention

The present invention relates to cell-based assays. The present invention relates to a method for on-chip functional screening assays of cellular responses. The present invention relates to a method for screening and pharmacological profiling of compounds modulating a cellular physiological response.

Background to the Invention

Within the pharmaceutical industry, there is a significant number of compounds, available in compound repositories, through the use of combinatorial chemistry. Hits from these compound libraries are identified through the use of high throughput screening (HTS). The primary function of HTS is to test various chemical compounds from their compound repository against multiple disease targets in many different biological assays. Traditional HTS utilizes 96-well microtiter plates. There is a large push in the pharmaceutical industry to miniaturize these microplate assays to reduce cost, reduce waste, and speed up timelines. There has been a change from the 96-well format to higher well densities such as 384- and 1536-well formats, however these can present challenges in relation to liquid handling, signal detection instrumentation, and assay technology. In addition, typical difficulties encountered when using microtiter plate based screening assays include e.g. (i) differential growth and/or gene expression of identical clones in separate wells of the microtiter plate and (ii) differential stress exposure (e.g. heat treatment, humidity) across the plate.

Efforts have been made towards resolving the aforementioned difficulties. For example, WO 99/35496 provides a method and apparatus for high density format screening for bioactive molecules with a much simplified technique for test compound delivery to a layer of cells, i.e. without the need of complicated fluid handling. In the presented method, up to 6144 test compounds may be simultaneously screened for bioactivity.

Nevertheless, there is a need for even higher density cell-based functional biological assays which remain one of the most difficult types of assays to miniaturize due to the limitations of delivering microliter quantities of cells consistently without shearing the cells or activating stress responses of the cells themselves, therefore interfering with the biological assay.

With the advent of combinatorial chemistry approaches to identify pharmacologically useful compounds, it is increasingly evident that there is a need for methods and apparatuses at microarray levels, capable of performing high throughput characterization of pharmacological profiles and corresponding potencies of the compounds in synthesized combinatorial libraries.

Microarrays of living cells could provide a shortcut to the development of safer drugs and a fuller understanding of e.g. the human genome. As an example, the Whitehead Institute for Biomedical Research in Cambridge developed microarrays of living cells for high-throughput analysis of gene function in mammalian cells (Nature, Vol. 411, 3 May 2001). While said technology is highly effective, there are, however, a number of limitations to its use including the relative high reagent quantities needed, of which a substantial amount is never in contact with the array and is therefore wasted and the requirement of cumbersome and time-consuming handlings.

As will be appreciated in the art, there is a continuous need for improved methods which overcome the aforementioned disadvantages.

It is therefore an object of the present invention to provide a highly efficient and cost-effective method for integrated cell-based assays using microarrays.

It is a further object of the present invention to provide a method for high-throughput cell-based assays requiring minimal amounts of sample and reagents.

Summary of the Invention

The present invention relates to a method for screening cellular responses of cellular components comprising:

(a) providing cellular components on the surface of a substrate, said substrate having immobilized thereon an array of detector molecules;

(b) delivering test compounds to positions on the substrate corresponding to the arrayed detector molecules on the surface of said solid substrate;

(c) incubating said test compounds with said cellular components on the surface of the solid support, under conditions allowing the induction of cellular responses;

(d) assaying said cellular responses; and,

(e) identifying and characterizing the cellular responses induced by said test compounds.

The present invention further discloses uses of the above method according to the invention.

The present invention provides for a miniaturization to microarray format of cell-based assays, thereby increasing throughput, while decreasing the volumes of reagents and test compounds.

Due to the flow-through characteristics of the device used in said method, the present method further provides for high speed and efficient analysis.

The method according to the invention allows for a non-invasive on-chip culturing of a confluent layer of cells or cellular components, said layer growing under equal conditions across the surface of the solid substrate, and said cells or cellular components being in contact with the culturing medium only for the time necessary to obtain a desired density.

Additional features and advantages of the invention will be set forth in the detailed description which follows, and in part will be apparent from the description, or may be learned by practice of the invention. The objectives and other advantages of the invention will be realized and attained by the process particularly pointed out in the written description and appended claims.

Detailed Description of the Invention

Before the present method and solutions used in the method are described, it is to be understood that this invention is not limited to particular methods, components, or solutions described, as such methods, components, and solutions may, of course, vary. It is also to be understood that the terminology used herein is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein

may be used in the practice or testing of the present invention, the preferred methods and materials are now described.

In this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise.

Performing a screen on many thousands of compounds requires parallel handling and processing of many compounds and assay component reagents. Standard high throughput screens use mixtures of compounds and biological reagents along with some indicator compound loaded into arrays of wells in standard microtiter plates.

The present invention relates to a large-scale miniaturization comprising use of a solid substrate to which a multitude of molecules are attached in predefined regions to form a microarray.

Three different components are involved in the cellular arrays according to the present invention: cellular components, test compounds and receptor molecules. The present invention provides a versatile integrated cellular-based assay wherein a number of test formats are envisaged.

In an array of cellular components, islands of different cells are grown or deposited on the substrate in an array format. Subsequently the whole array is exposed to one (or a limited number) of test compounds and finally exposed to one (or a limited number) of receptor molecules (possibly present in the substrate) if necessary after lyses. As such, this test format allows the screening of an array of different cellular components for responses induced by a particular test compound, detected with a particular detector molecule. Said detector molecule may be provided subsequent to the incubation of the test compound with the cellular compounds or may have been introduced within the substrate prior to contact of the substrate with the cellular components.

The terms "detector molecule", "receptor molecule" and "discriminator molecules" may be used interchangeable and refer, in the context of the present invention, to molecules which allow the detection of a cellular response.

In a test substance array a homogeneous layer of a cellular component is locally, at predefined regions, treated, by spotting, with various test substances. After treatment, cellular responses may be detected with a particular detector molecule. Said detector molecule may be provided subsequent to the incubation of the test compound with the cellular compounds or may have been introduced within the substrate prior to contact of the substrate with the cellular components.

In a detector array, an array of different receptor molecules are contacted with a homogeneous layer of cellular components which are treated with a particular test compound (or a few after each other). Cellular responses are monitored by detecting excretion products by the receptor molecules or by detecting intracellular products through binding to the receptor molecules after lysis of the cellular components.

The nature and geometry of the solid substrate will depend upon a variety of factors, including, among others, the type of array and the mode of attachment. Generally, the substrate may be composed of any material which will permit cell culturing and immobilization of the desired molecules and which will not melt or otherwise substantially degrade under the conditions used to perform cell-based assays. In addition, where covalent immobilization is contemplated, the substrate should be activatable with reactive groups capable of forming a bond, which may be covalent, with the molecule to be immobilized.

A number of materials suitable for use in substrates as used in the present invention have been described in the art. Exemplary suitable substrates in the present invention comprise materials including acrylic, acrylamide, methylene-bis-acrylamide, dimethylaminopropylmethacrylamide, styrenemethyl methacrylate copolymers, ethylene/acrylic acid, acrylonitrile-butadienestyrene (ABS), ABS/polycarbonate, ABS/polysulfone, ABS/polyvinyl chloride, ethylene propylene, ethylene vinyl acetate (EVA), nitrocellulose, polycarylonitrile (PAN), polyacrylate, polycarbonate, polybutylene terephthalate (PBT), polyethylene terephthalate (PET), polyethylene (including low density, linear low density, high density, cross-linked and ultra-high molecular weight grades), polypropylene homopolymer, polypropylene copolymers, polystyrene (including general purpose and high impact grades), polytetrafluoroethylene (PTFE), fluorinated ethylene-propylene (FEP), ethylene-tetrafluoroethylene (ETFE), perfluoroalkoxyethylene (PFA), polyvinyl fluoride (PVF), polyvinylidene fluoride (PVDF), polychlorotrifluoroethylene (PCTFE), polyethylene-

chlorotrifluoroethylene (ECTFE), polyvinyl alcohol (PVA), silicon styreneacrylonitrile (SAN), styrene maleic anhydride (SMA), and glass. Further exemplary suitable substrates comprise mixtures of two or more of the above-mentioned materials.

Other exemplary suitable materials for the manufacture of substrates in the present invention include metal oxides. Metal oxides provide a support having both a high channel density and a high porosity, allowing high density arrays comprising different first binding substances per unit of the surface for sample application. In addition, metal oxides are highly transparent for visible light. Metal oxides are relatively cheap substrates that do not require the use of any typical microfabrication technology and, that offer an improved control over the liquid distribution over the surface of the substrate, such as electrochemically manufactured metal oxide membrane. Metal oxide membranes having through-going, oriented channels may be manufactured through electrochemical etching of a metal sheet. Metal oxides considered are, among others, oxides of tantalum, titanium, and aluminum, as well as alloys of two or more metal oxides and doped metal oxides and alloys containing metal oxides. The metal oxide membranes are transparent, especially if wet, which allows for assays using various optical techniques. Such membranes have oriented through-going channels with well-controlled diameter and useful chemical surface properties. WO 99/02266, which describes the use of Anopore™, is exemplary in this respect, and is specifically incorporated in the present invention.

Accordingly, in one embodiment of the present invention, the solid substrate is a porous solid support.

In a further embodiment, the solid substrate as comprised in the steps of the method of the present invention is a flow-through solid support.

In a further embodiment, the solid substrate as comprised in the steps of the method of the present invention is a metallo-oxide substrate.

In a further embodiment, the solid substrate as comprised in the steps of the method of the present invention is an aluminum-oxide substrate.

The term "cellular components" as used throughout the present specification refers to whole intact viable cells including, e.g. prokaryotic cells; as well as cell components such as vesicles and organelles; as well as sectioned material such as tissue sections; as well as fixed cells; as well as microscopic multicellular organisms such as, e.g., nematodes; and others.

According to the present invention, the surface of said solid substrate may be contacted, by direct deposit thereon, with an inoculum of cellular components. Said inoculum may be a liquid formulation comprising said components and an appropriate growth medium.

The final inoculum, however, may also be disposed of any growth medium and comprise preservers instead such as glycerol (e.g. bacterial cultures). Accordingly, cellular components may be preserved on the substrate for analysis later on; i.e. cellular components may be on the substrate under preserving conditions such as in glycerol or other suitable medium or lyophilised. The term "preserving condition" refers to a condition to keep the cellular components alive and/or intact and free from decay.

Alternatively, a culture of cellular components may be incubated for growth until the exponential phase with respect to their growth curve is reached, followed by deposition of an aliquot of said culture directly on the substrate.

Accordingly, in one embodiment of the present invention a method is provided wherein said providing of cellular components on the surface of a substrate is by a deposit directly on said substrate of an inoculum or a culture.

As will be appreciated by a person skilled in the art, established protocols are available for the culture of diverse cell types. Such protocols may require the use of specialized coatings and selective media to enable cell growth and the expression of specialist cellular functions. None of such protocols is precluded from use with the method of the present invention.

In the present invention, nutrients may be provided to the surface of the solid substrate from underneath or from above and through the pores of said solid substrate.

Said nutrients may be provided with a growth medium to culture the cells. Said growth medium may be any conventional medium suitable for growing the host cells, such as

minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g.. in catalogues of the American Type Culture Collection). The media are prepared using procedures known in the art.

The provision of the nutrients to the substrate for growth of the cellular components is under aseptic conditions. Said aseptic conditions may be accomplished by working in a laminar flow bench or by placing a cover on the substrate; i.e. on the side of deposit or growth of the cellular components.

The method according to the present invention may also be applicable to sectioned material which may be directly positioned in contact with the substrate.

If required for downstream assays, e.g. immuno-fluorescent detection, cells may be fixed onto the surface of the solid substrate, e.g. by chemical fixation. Typically, the preferred fixative will depend upon whether the cellular response manifests or the molecule of interest is localized at the cell's surface or within the cell. For example, some fixation methods (such as methanol or acetone fixation) are not usually used on cells that will need to be permeabilized (e.g. examination of intracellular antigens).

Various fixation protocols for various cell types for various assays are well known in the art; e.g. mammalian cells may be contacted with a fixative such as phosphate-buffered saline (PBS) with 3.7% paraformaldehyde and 4.0% sucrose.

The term "cellular component" as used in the present invention encompasses any cell types that can be cultured on standard tissue culture ware. Both adherent and non-adherent cell types may be used. A "cellular component" as used in the present invention means any cell which allows the detection of a response upon exposure or treatment to/with a test compound. A cellular component according to the present specification may be a wild type, a mutant or a transformed or transfected cell and may therefore afford the subsistence or lodgment of a non-host substance; said non-host substance may be viable such as e.g. a parasite or non-viable such as e.g. a vector and my be stably or transiently present in said host cell. A cell has been transfected by exogenous or heterologous genetic material when such material has been introduced inside the cell. A cell has been transformed by exogenous

or heterologous genetic material when the transfected material effects a cellular change, e.g. a phenotypic change. Usually, the transforming genetic material should be integrated into the cell's chromosomal DNA making up its genome. Integration of transforming genetic material including vector DNA into the host chromosome may occur by homologous or non-homologous recombination. Further, a "cellular component" as used in the present specification encompasses any progeny of a parent cell which is not identical to the parent cell due to mutations that occur during replication.

Useful cells include prokaryotes and eukaryotes such as mammalian cells including hybridoma cells, insect cells, plant cells, yeast cells, and protist cells comprising protozoa, algae and fungal cells. Mammalian cells may be derived from any recognized source with respect to species (e.g. human, rodent, simian), tissue source (brain, liver, lung, heart, kidney, skin, muscle) and cell type (e.g. epithelial, endothelial). In addition, cells which have been transfected with recombinant genes may also be cultured using the present invention.

Suitable cell lines may be comprised within e.g. the American Type Culture Collection and the German Collection of Microorganisms and Cell Cultures.

In one embodiment of the present invention, the cellular components are selected from the group comprising mammalian cells, insect cells, yeast cells, plant cells, and microbial cells including bacterial and fungal cells, cellular vesicles, cellular organelles, tissue sections, and whole microscopic organisms including nematodes.

Non-limiting examples of useful mammalian cell lines include animal and human cell lines such as Chinese hamster ovary (CHO) cells, Chinese hamster lung (CHL) cells, baby hamster kidney (BHK) cells, COS cells, HeLa cells, THP cell lines and TAg Jurkat cells.

Suitable insect cell lines include but are not limited to *Lepidoptera* cell lines such as *Spodoptera frugiperda* cells (e.g. Sf9, Sf21) and *Trichoplusia ni* cells (e.g. High Five™, BTI-Tn-5B1-4).

Non-limiting examples of fungal cells useful in the present invention include the phyla *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota* as well as the *Oomycota* and all mitosporic fungi. Representative groups of *Ascomycota* include, e.g., *Neurospora*,

Eupenicillium (or *Penicillium*), *Emericella* (or *Aspergillus*), *Eurotium* (or *Aspergillus*), and the true yeasts listed above. Examples of *Basidiomycota* include mushrooms, rusts, and smuts. Representative groups of *Chytridiomycota* include, e.g., *Allomyces*, *Blastocladiella*, *Coelomomyces*, and aquatic fungi. Representative groups of *Oomycota* include, e.g., saprolegniomycetous aquatic fungi (water molds) such as *Achlya*. Examples of mitosporic fungi include *Aspergillus*, *Penicillium*, *Candiaa*, and *Alternaria*. Representative groups of *Zygomycota* include, e.g., *Rhizopus* and *Mucor*.

Fungal cells may be yeast cells. Non-limiting examples of useful yeast cells include ascosporogenous yeast (*Endomycetales*), basidiosporogenous yeast, and yeast belonging to the *Fungi Imperfecti* or *Deuteromycota* (*Blastomycetes*). The ascosporogenous yeasts are divided into the families *Spermophthoraceae* and *Saccharomycetaceae*. The latter is comprised of four sub-families, *Schizosaccharomycoideae* (e.g., genus *Schizosaccharomyces* including *S. pombe*), *Nadsonioideae*, *Lipomycoideae*, and *Saccharomycoideae* (e.g., genera *Pichia* including *P. pastoris*, *P. guillermondii* and *P. methanolio*), *Kluyveromyces* including *K. lactis*, *K. fragilis* and *Saccharomyces* including *S. carlsbergensis*, *S. cerevisiae*, *S. diastaticus*, *S. douglasii*, *S. kluyveri*, *S. norbensis* or *S. oviformis*). The basidiosporogenous yeasts include the genera *Leucosporidim*, *Rhodosporidium*, *Sporidiobolus*, *Filobasidium*, and *Filobasidiella*. Yeasts belonging to the *Fungi Imperfecti* are divided into two families, *Sporobolomycetaceae* (e.g., genera *Sporobolomyces* and *Bullera*) and *Cryptococcaceae* (e.g., genus *Candida* including *C. maltose*). Other useful yeast host cells are *Hansehula polymorpha*, *Yarrowia lipolytica*, *Ustilgo maylis*.

Fungal cells may be filamentous fungal cells including all filamentous forms of the subdivision *Eumycota* and *Oomycota*. Filamentous fungi are characterized by a vegetative mycelium composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligatory aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative. In a more preferred embodiment, the filamentous fungal host cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, and *Trichoderma* or a teleomorph or synonym thereof.

Useful microorganism cells may be unicellular, e.g. a prokaryotes, or non-unicellular, e.g. eukaryotes. Useful unicellular cells are aerobic bacterial cells such as gram positive bacteria including, but not limited to, the genera *Bacillus*, *Sporolactobacillus*, *Sporocarcina*, *Filibacter*, *Caryophanum*, *Arthrobacter*, Staphylococcus, Planococcus, *Micrococcus*, *Mycobacterium*, Nocardia, Rhodococcus; or gram negative bacteria including, but not limited to, the genera *Acetobacter, Gluconobacter, Frateuria, Alcaligenes, Achromobacter, Deleya, Amoebobacter, Chromatium, Lamprobacter, Lamprocystis, Thiocapsa, Thiocystis, Thiodictyon, Thiopedia, Thiospirillum, Escherichia, Salmonella, Shigella, Erwinia, Enterobacter, Serratia, Legionella, Neisseria, Kingella, Eikenella, Simonsiella, Alysiella, Nitrobacter, Nitrospina, Nitrococcus, Nitrospira, Pseudomonas, Xanthomonas, Zoogloea, Fraturia, Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium, Rickettsia, Rochalimaea, Ehrlichia, Cowdria, Neorickettsia, Treponema, Borrelia, Vibrio, Aeromonas, Plesiomonas, Photobacterium, Brucella, Bordetella, Flavobacterium, Francisella, Chromobacterium, Janthinobacterium, and Iodobacter.*

Suitable plant cells for use in the present invention include dicotyledonous plant cells, examples of which are tobacco, potato, tomato, and leguminous (e.g. bean, pea, soy, alfalfa) cells. It is, however, contemplated that monocotyledonous plant cells, e.g. cereal plant cells, may be equally suitable.

Delivery of test compounds, cellular components or detector molecules to predefined regions on the substrate may be accomplished by using a liquid handling device but may equally be accomplished by manual handling.

Accordingly, a liquid handling device may be positioned on the substrate, wherein said liquid handling device may be a high precision x-y-z pipettor or inkjet printer containing 1 or more channels through which liquid can be dispensed, sequentially or in parallel, to positions corresponding to arrayed molecules on the surface of the solid substrate. Alternatively, a superposing mask comprising transversal holes may be superposed onto the substrate, wherein said superposing is such that each transversal hole in said mask correspond to an arrayed molecule on the surface of said solid substrate.

Said delivering may be by means of contact or non-contact spotting. The term "contact spotting" or "contact force" as used in this specification means a direct surface contact between a printing substrate and a delivery mechanism that may contain an array of

tweezers, pins or capillaries that serve to transfer or deliver any content within the delivery mechanism to the surface by physically tapping said array of tweezers, pins or capillaries on the surface. Further, a superposing mask may be positioned on the cells-containing solid support whereby the content of the wells as formed by the filled holes in the mask is passively delivered onto said cells by capillary actions when pressing the mask onto the chip. As used in the present specification, a mask acts as a barrier to the passage of a reagent. Typically, a pattern of holes in the mask allows selective passage of reagent and results in a corresponding pattern of reagent deposition on a surface placed behind/below the mask.

Alternatively, the test compounds may also be delivered or spotted through ink-jet printing technology, a non-contact technology in which reactants are sprayed onto the surface using technology adapted from computer ink- jet printers. The ink- jet method is sometimes called indirect because the reactants are sprayed onto the surface rather than being directly placed. Ink- jet methods may be capable of producing smaller spots, and because they avoid physical contact with the surface may prove to be more reliable.

Useful ink-jet printing methodologies may include continues and drop-on-demand ink-jet methods. Most suitable ink-jet printing methods are drop-on-demand ink-jet methods, examples of which include piezoelectric and electrostatic ink-jet systems.

Further useful in the present invention are spotting robots or liquid handling devices. Most spotting robots or liquid handling devices use an X-Y-Z robot arm (one that can move in three dimensions) mounted on an anti-vibration table. Pins held by the arm are dipped into a first microtiter plate to pick up the fluid (e.g. test compound solution) to be delivered. The tips of the pins are then moved to the solid support surface and allowed to touch the surface only minimally; the test compound solution is then transferred. The pins are then washed and moved to the next set of wells and test compounds. This process is repeated until hundreds or thousands of test compounds are deposited. Solid pins, quills, and pin- and- ring configurations of pins may be useful.

Accordingly, in one embodiment of the present invention, delivery of test compounds is by a means chosen from the group comprising a delivery mask, a high precision x-y-z pipettor, inkjet printer, and manual handling.

In a further embodiment of the present invention, delivery of test compounds is by a means of a high precision x-y-z pipettor or inkjet printer.

In one embodiment of the present invention, the delivery of test compounds to the cells-containing support is by means of a contact force.

In a further embodiment of the present invention, the delivery of test compounds to the cells-containing support is by means of a contact force which may be a capillary force or a piezo-electric force.

The present invention provides a method for screening and the pharmacological profiling of compounds modulating a cellular response, e.g. a physiological response and/or the activities of cells. A variety of effects caused by the compounds to be screened may be detected and quantitatively characterized according to the present invention. These effects include but are not limited to changes in intracellular concentration of ionized calcium, cAMP differences (e.g. due to metabolic activation or inactivation), pH, temperature, NO, and transmembrane potential, intracellular Ca-, K- or Na-fluxes in or out of the cell and other physiological and biochemical characteristics of living cell which can be measured by a variety of conventional means, for example using specific fluorescent, luminescent or color developing dyes.

The present invention also includes methods of screening for agonist or antagonist activity of drugs, methods of characterizing their potency profiles, methods of identifying the receptor expression pattern of cell membrane ("receptor fingerprinting"), methods of determining toxicity profiles for the compounds (e.g. toxicological responses, CYP-450, HERC), apoptosis, cellular necrosis, cell mutation processes such as e.g. carcinogenesis, drug induced protein protein interactions detectable using fluorescence resonance energy transfer (FRET) or bioluminescent resonance energy transfer (BRET), ADME (adsorption, distribution, metabolism and elimination) or any other cellular responses. The plurality of cellular responses includes a cellular response selected from the group consisting of activation or inhibition of a receptor mediated response, activation or inhibition of an ion channel, activation or inhibition of a non-selective pore, activation or inhibition of a second messenger pathway at a point downstream of a receptor or channel, activation or inhibition of apoptosis, and activation or inhibition of cellular necrosis, and cellular toxicity.

The method of the present invention may also be used to perform biochemical analyses, such as Western analyses, Northern analyses, detection of single nucleotide polymorphisms (SNPs), detection of enzymatic activities, or molecular assembly assays.

According to the method of the present invention, the ability and potency of substances to act as agonists or antagonists against receptors, ion channels, ion pumps, and ion transporters localized on a cell surface membrane may be detected, evaluated and characterized. These molecular assemblies work in concert to maintain intracellular ion homeostasis. Any changes in the activity of these systems would cause a shift in the intracellular concentrations of ions and consequently to the cell metabolic response.

Ion pumps act to maintain transmembrane ion gradients utilizing ATP as a source of energy. Examples of ion pumps are: Na^+ /K^+-ATPase maintaining transmembrane gradient of sodium and potassium ions, Ca^{2+} -ATPase maintaining transmembrane gradient of calcium ions and H^+-ATPase maintaining transmembrane gradient of protons.

Ion transporters use the electrochemical energy of transmembrane gradients of one ion species to maintain gradients of other ion counterpart. For example, the Na^+/Ca^{2+} -exchanger uses the chemical potential of the sodium gradient directed inward to pump out calcium ions against their chemical potential.

Ion channels, upon activation, allow for the ions to move across the cell membrane in accordance with their electrochemical potential.

Accordingly, in one embodiment of the present invention, a method as described herein is provided, wherein cellular responses are chosen from the group comprising chemically induced or physiological events in the cell including lysis, apoptosis, growth inhibition, and growth promotion; production, secretion, and surface exposure of a protein or other molecule of interest by the cell; membrane surface molecule activation including receptor activation; trans-membrane ion transports; and transcriptional regulations.

Molecules of interest which may be monitored may be any molecule of biological origin, non-limiting examples of which are peptides, polypeptides, proteins, enzymes, post-translational modified polypeptides such as lipopeptides or glycosylated peptides, antimicrobial peptides or

molecules, primary or secondary metabolites such as alginates, small organic molecules, molecules having pharmaceutical properties, etc.

In a further embodiment of the present invention, a method is provided wherein said molecule of interest is selected from the group comprising peptides including lipopeptides, glycosylated peptides and antimicrobial peptides, polypeptides, proteins, enzymes, antimicrobial molecules, primary and secondary metabolites, and small organic molecules including pharmaceutical molecules.

In one embodiment of the present invention, a method is provided, wherein said test compound is a drug or any compound which might become a drug.

The number of possible test compounds runs into millions. Commercially available compound libraries including peptides, proteins, sugars, etc. may be obtained from, e.g., ArQule, Pharmacopeia, Graffinity, Panvera, and Oxford.

In a particular embodiment of the present invention, said test compound is a drug selected from a chemical or natural drug candidate library.

In one embodiment of the present invention, a method as described is provided wherein assaying of cellular responses is by:

(a) providing a detection agent to the cellular components;
(b) washing off excess of unincorporated detecting agent; and,
(c) detecting the presence or absence of a change in detectable signal, the presence of a change in detectable signal indicating a cellular response.

Alternatively, label free detection of cellular responses may be envisaged by e.g. calorimetric measurements. This allows the measurement of e.g. metabolic activities in a cell by detection with for example a sensitive IR camera.

In one embodiment of the present invention, cellular responses are assayed in whole broth or cell culture medium, in isolated cells such as pelleted cells, in supernatant of the cellular components, or in lysate of the cellular components.

In one embodiment of the present invention, detector molecules are selected from the group comprising nucleic acids including modified analogues thereof, peptides, proteins, and antibodies including antibody fragments, and specific dyes.
Non-limiting suitable examples of specific dyes are well known in the art and include Fluo-3, Fluo-4, and Ca –dyes such as e.g. Calcium Green-1.

Cells or cellular components may be modified with luminescent indicators for chemical or molecular cellular properties and may be analysed in a living state. Said indicators may be introduced into the cells before or after they are challenged with test compounds and by any one or a combination of a variety of physical methods, such as, but not limited to diffusion across the cell membrane, mechanical perturbation of the cell membrane, or genetic engineering so that they are expressed in cells under prescribed conditions. Live studies permit analysis of the physiological state of the cell as reported by the indicator during its life cycle or when contacted with a test compound such as a drug or other reactive substance.

Accordingly, in one embodiment of the present invention, providing a detection agent to the cellular components is prior to delivering of test compound thereby providing pre-labeled cellular components.

In one embodiment of the present invention, identifying the cellular responses is by luminescence.

In a further embodiment of the present invention, said luminescence is fluorescence.

Particularly useful fluorescent molecules include, by way of example and not limitation, fluorescein isothiocyanate (FITC), rhodamine, malachite green, Oregon green, Texas Red, Congo red, SybrGreen, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), 6-carboxy X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), cyanine dyes (e.g. Cy5, Cy3), BODIPY dyes (e.g. BODIPY 630/650, Alexa542, etc), green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), and the like, (see, e.g., Molecular Probes, Eugene, Oregon, USA).

Means for detecting signals in general are well known to those of skill in the art. Thus, for example, radiolabels may be detected using photographic film or scintillation counters, fluorescent markers may be detected using a photodetector to detect emitted illumination. Enzymatic labels are typically detected by providing the enzyme with an enzyme substrate and detecting the reaction product produced by the action of the enzyme on the substrate, and colorimetric labels are detected by simply visualizing the coloured label.

In one embodiment of the present invention, assaying is performed in real-time.

In another embodiment of the present invention, assaying is an end-point assaying.

The methods according to the present invention can be used particularly for monitoring induced cellular responses of host cells.

Additionally, the methods according to the present invention can be used particularly for functional screening of cellular responses upon assaying cellular components with test compounds.

The method and microarrays according to the present invention may further be found particularly suitable for screenings of arrays of e.g. antibiotics with mycoplasms.

As will be well appreciated in the art, methods and microarrays according to the present invention are also particularly suitable for combinatorial screenings.

It may be desirable to provide test compounds in solution at predefined regions within a substrate for screenings at a layer time-point. Microarrays as used in the method of the present invention are particularly suitable for the preservation of test compounds. If provided in solution, said test compounds may move freely during screenings procedures. This might be advantageous compared to affixed compound which might provoke a steric hindrance effect.

Accordingly, it is another object of the present invention to provide a microarray for performing a method according to the present invention wherein an array of test compounds

is provided within predefined regions, said test compounds are in liquid solution and not immobilized in the substrate.

It is yet another object of the present invention to provide a microarray for performing a method according to the present invention wherein an array of cellular components is provided in predefined regions on a substrate, said cellular components being conditioned for preservation on said substrate.

It is yet another object of the present invention to provide a microarray for performing a method according to the present invention wherein a cellular component is provided on a substrate, said cellular component being conditioned for preservation on said substrate.

In a one embodiment of the invention, a microarray as described herein is provided wherein an array of detector molecules is immobilized within the substrate.

In a further embodiment of the present invention, a microarray according to the present invention is provided wherein said array of detector molecules comprises a plurality of equal detector molecules or a plurality of different detector molecules.

In yet a further embodiment o-f the present invention, a microarray according to the present invention is provided wherein said condition is chosen from the group comprising, lyophilization and glycerol dissolution.

It is yet another object of the invention to provide a kit for performing a method according to the present invention comprising a microarray as described herein.

EXAMPLES

The following examples of the invention are exemplary and should not be taken as in any way limiting.

Example 1:

Antibodies against the heat shock proteins HSP90alpha, HSP90beta, PolyUBQ and beta-actin are spotted in an array format on an aluminium-oxide substrate (Anopore, Whatman) using standard protocols

Jurkat cells are grown under standard conditions in a CO_2 incubator to a density of 10^6 cells/ml. 25 µl of cell suspension is added to the array. The culture medium is removed by suction through the array using the standard equipment, and 50 µl of fresh medium is added. The cells are incubated under sterile conditions at 37 °C on the substrate for 24 hrs, or till a density of 10^7 cells/ml is reached. The temperature is raised to 43 °C for 15 min. The control is kept at 37 °C. The culture medium is removed by suction through the array. The cells are washed with 50 µl wash liquid. After removal of this liquid by suction through the array, 25 µl lysis buffer is added. After 15 min of lysis, the liquid is pumped back and forth through the array for 15 min with two cycles, of up and down flow, per minute. The liquid is removed by suction, the array is washed with 25 µl of wash buffer, which is removed by suction. 20 µl of a mixture of anti-heatshock antibodies (anti-HSP90alpha, anti-HSP90beta, anti-PolyUBQ and anti-beta-actin, all labeled with fluorescein), is added and pumped through the array for 15 minutes at two cycles per minute. Images are captured every two minutes. In the case the background is too high, the antibody solution is removed from the array and the array is washed with wash buffer. Images are taken. Data will be analysed using standard software.

Antibodies may be monoclonals, polyclonals, single chain antibodies, whereby those spotted on the substrate recognise another epitope than those added for detection of bound antibodies.

Example 2. Induction of Cytochrome P450 Isoenzyme 1A (CYP1A).

Ethoxyresorufin is chemically modified with a crosslinker and spotted on a aluminium-oxide substrate using spotting technology as well known in the art.

Hepatocytes are prepared as described by van 't Hoen *et al.* (2000). Viability is judged by tryptan blue exclusion. 50 µl of cell suspension at a cell density of 80000 cells/ml is added to the substrate. The culture medium is removed by suction throughh the substrate using standard equipment. To allow adherence, the cells are initially cultured for 3.5 hr in DMEM containing 10 % (v/V0 fetal calf serum, 140 mU ml^{-1} insulin, 2 mM L-glutamine, 100 U ml^{-1}

penicillin, and 100 microgram ml^{-1} streptomycin in a humidified CO_2 atmosphere at 37 °C. Thereafter, non-adhering cells are washed away by pipetting the medium away. The incubation medium is changed to serum-free DMEM, containing 0.2 % (w/.v) BSA, 140 mU ml^{-1} insulin, 2 mM L-glutamine, 200 U ml^{-1} penicillin, and 200 microgram ml^{-1} streptomycin. The hepatocytes will form a confluent layer within 1 day. 24 hrs after application of the cells to the array, most of the medium is removed by suction, taking care not to dry the cells.

Benzo[a]pyrene, dexamethasone, phenobarbital, hexobarbital, debrisquine, aniline, midazolam (concentration ranging from 1 to 100 µM in DMSO) are applied on specific locations (arrayed) onto the cell layer with piezo inkjet spotting technology. 30 min after application of the inducers, 25 microliter of cuture medium is added.

At different time points the medium is removed by suction and 5 µl lysis buffer is added. 2 µl of a reaction solution is added and the lysate is pulled into the array. Development of fluorescence by liberation of resorufin in the pores is monitored using the standard equipment, and capturing images every 10 sec.

These compounds induce different isoenzymes of cytochrome c. Some are very active in converting ethoxyresorufin, others hardly or not at all. By lysis, cyt P450 should diffuse out of the cell, but this may be difficult since it is a membrane bound enzyme that may need coenzymes and cofactors.

Alternatively, it may not be necessary to couple ethoxyresorufin to the substrate, addition and uptake by the cells may allow monitoring of activity in situ, before it diffuses away.

During this enzymatic reaction no pumping is done to prevent the product from diffusing away from the reaction site.

Reference

P.A.Chr 't Hoen *et al.* Selective induction of cytochrome P450 3A1 by dexamethason in cultured rat hepatocytes. Analysis with a novel reverse transcriptase-polymerase chain reaction assay. Biochemcal Pharmacology, vol
60 pp 1509-1518 (2000)

Claims

1. A method for screening of cellular responses of cellular components comprising:
   (a) providing cellular components on the surface of a substrate, said substrate having immobilized thereon an array of detector molecules;
   (b) delivering test compounds to positions on the substrate corresponding to the arrayed detector molecules on the surface of said solid substrate;
   (c) incubating said test compounds with said cellular components on the surface of the solid support, under conditions allowing the induction of cellular responses;
   (d) assaying said cellular responses; and,
   (e) identifying and characterizing the cellular responses induced by said test compounds.

2. A method according to claim 1, wherein said solid substrate is a porous solid substrate.

3. A method according to any of claims 1 to 2, wherein said solid substrate is a flow-through solid substrate.

4. A method according to any of claims 1 to 3, wherein said providing of cellular components on the surface of a substrate is by a deposit directly on said substrate of an inoculum or a culture.

5. A method according to any of claims 1 to 4, wherein said delivering of test compounds is by means of contact force.

6. A method according to claim 5, wherein said contact force is a capillary force or a piezo-electric force.

7. A method according to any of claims 1 to 6, wherein said assaying of cellular responses is by:
   (a) providing a detection agent to the cellular components;
   (b) washing off excess of unincorporated detecting agent;
   (c) detecting the presence or absence of a change in detectable signal, the presence of a change in detectable signal indicating a cellular response.

8. A method according to any of claims 1 to 7, wherein said cellular response is assayed in whole broth or cell culture medium, in isolated cells such as pelleted cells, in supernatant of the cellular components, or in lysate of the cellular components.

9. A method according to any of claims 1 to 8, wherein said delivery of test compounds is by a means chosen from the group comprising a delivery mask, a high precision x-y-z pipettor, inkjet printer, and manual handling.

10. A method according to claim 9, wherein said delivery of test compounds is by means of a high precision x-y-z pipettor or inkjet printer.

11. A method according to any of claims 1 to 10, wherein said identifying of the cellular responses is by luminescence.

12. A method according to claim 11, wherein said luminescence is fluorescence.

13. A method according to any of claims 1 to 12, wherein said cellular components are selected from the group comprising mammalian cells, insect cells, yeast cells, plant cells and microbial cells including bacterial and fungal cells, including cellular vesicles, cellular organelles, tissue sections, and whole organisms including nematodes.

14. A method according to any of claims 1 to 13, wherein said detector molecules are selected from the group comprising nucleic acids including modified analogues thereof, peptides, proteins, and antibodies including antibody fragments, and specific dyes.

15. A method according to any of claims 1 to 14, wherein said cellular responses are chosen from the group comprising chemically induced or physiological events in the cell including lysis, apoptosis, growth inhibition, and growth promotion; production, secretion, and surface exposure of a protein or other molecule of interest by the cell; membrane surface molecule activation including receptor activation; trans-membrane ion transports; and transcriptional regulations.

16. A method according to claim 15, wherein said molecule of interest is selected from the group comprising peptides including lipopeptides, glycosylated peptides and antimicrobial

peptides, polypeptides, proteins, enzymes, antimicrobial molecules, primary and secondary metabolites, and small organic molecules including pharmaceutical molecules.

17. A method according to any of claims 1 to 16, wherein said test compound is a drug or any compound which might become a drug.

18. A method according to claim 17, wherein said test compound is a drug selected from a chemical or natural drug candidate library.

19. A method according any of claims 1 to 18, wherein said solid substrate is a metallo-oxide substrate.

20. A method according to claim 19, wherein said solid substrate is an aluminum-oxide substrate.

21. A method according to any of claims 1 to 20, wherein said assaying is performed in real-time.

22. Method according to any of claims 1 to 21, wherein said assaying is an end-point assaying.

23. A method according to claim 7, wherein said providing a detection agent to the cellular components is prior to delivering of test compound thereby providing pre-labeled cellular components.

24. Use of a method according to any of claims 1 to 23, for monitoring induced cellular responses of host cells.

25. Use of a method according to any of claims 1 to 23, for functional screening of cellular responses upon assaying host cells with test compounds.

26. A microarray for performing a method according any of claims 1 to 23, wherein an array of test compounds is provided within predefined regions, said test compounds are in liquid solution and not immobilized in the substrate.

27. A microarray for performing a method according any of claims 1 to 23, wherein an array of cellular components is provided in predefined regions on a substrate, said cellular components being conditioned for preservation on said substrate.

28. A microarray for performing a method according any of claims 1 to 23, wherein a cellular components is provided on a substrate, said cellular component being conditioned for preservation on said substrate.

29. A microarray according to claim 27 or 28, wherein an array of detector molecules is immobilized within the substrate.

30. A microarray according to claim 29, wherein said array of detector molecules comprises a plurality of equal detector molecules or a plurality of different detector molecules.

31. A microarray according to claim 27 or 28, wherein said condition is chosen from the group comprising lyophilization and glycerol dissolution.

32. A kit for performing a method according to any of claims 1 to 23, comprising a microarray according to any of claims 26 to 31.

Abstract

The present invention relates to methods for screening of cellular responses of cellular components comprising:

(a) providing cellular components on the surface of a substrate, said substrate having immobilized thereon an array of detector molecules;

(b) delivering test compounds to positions on the substrate corresponding to the arrayed detector molecules on the surface of said solid substrate;

(c) incubating said test compounds with said cellular components on the surface of the solid support, under conditions allowing the induction of cellular responses;

(d) assaying said cellular responses; and,

identifying and characterizing the cellular responses induced by said test compounds. The present invention further relates to the uses of said methods as well as microarrays and kits for carrying out said methods.